FIGURE 1: FRONT COVER AD

ASSESSING THE EFFECT OF HUMIC ACIDS ON GERMINATING A NATIVE AMERICAN PLANT

FINAL TECHNICAL REPORT

BY

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SUMMARY

Slickspot peppergrass (*Lepidium papilliferum*) is a reported "rare" ephemeral endemic plant found in the United States in Southwest Idaho's high desert (Idaho Natural Heritage Program). The main objective of this research was to identifying and evaluating the effects of several naturally occurring humic acids (HAs) in Idaho soils on seed germination and seedling early-growth of slickspot peppergrass, and their possible relations to the compositional, structural and functional characteristics of humic acids. For this purpose, a total of n. 61 HAs isolated from various layers of a number of Idaho soils were characterized for their elemental (C, H, N, S, O) composition and structural and functional properties by using Fourier transform infrared (FT IR), bi-dimensional (emission-excitation matrix (EEM)) fluorescence and electron spin resonance (ESR) spectroscopies. The main germination and early plant growth experiments were then performed using three concentrations of: (a) two HAs originated from two Idaho soils (sites # 4 and #5) (HA4 and HA5) (each HA sample was obtained by mixing three corresponding HA subsamples from three layers (0-1", 1-2", and 2-3") of the two Idaho soils); and (b) three HAs isolated from three mixed layers labeled "silt in/out", "vesicular in/out", and "clay in/out", of an Idaho soil (soil # 4, Series II) (HA4-IIs, HA4-IIv, and HA4-IIc).

A preliminary objective was to test: (a) four well characterized standard HAs originated from (1) an Orthic Podzol under a coniferous forest (OHA); (2) the mollic horizon of the Elliot silt loam soil sampled near Joliet, IL, USA, (SHA); (3) the Pahokee peat sampled at Ocachobee in the Everglades, FL, USA, (PHA); and (4) a leonardite sampled at the Gascoyne mine, Bowman County, ND, USA, (LHA); and (b) three additional plant species, western wheatgrass and bluebunch wheatgrass (*Pseudoroegneria spicata*) and basin wildrye (*Leymus cinereus*).

Seed germination was performed in Petri dishes added with solutions of each HA at each concentration or with bidistilled water (control) and kept in the dark in a thermostated chamber at the temperature of 24 °C. Germination % and primary shoot and root lengths were measured on germinated seeds collected after 10, 9 or 6 days. All experiments were conducted in four replicates. Tentative germination experiments with slickspot peppergrass seeds were unsuccessful, thus a number of seed preliminary treatments and conditions were tested to improve germination. Among these the most appropriate pretreatment was to germinate seeds previously scraped, soaked in distilled water for 24 h, and pierced with a steel needle

The plant early growth experiment was performed on germinated seeds on Nitch nutrient solution, in the absence (control) or presence of each HA at each concentration at a pH adjusted at 6.4 or 7.3, in a Fitotron growth chamber for 30 or 24 days with a photoperioed of 12 h, a temperature of 25°C, and a humidity of 74%. At the end of the experiments, the solution pH and length and fresh and dry weight of roots and shoots were measured. All experiments were conducted in four replicates. All germination and early growth data were analysed statistically by one-way analysis of variance (ANOVA).

In general, germination and early growth results obtained both in the preliminary and main experiments are quite variable, and sometime contrasting, on dependence of the kind of seed and origin and concentration of HA used. Although no definite trend can be observed for the germination and early growth parameters measured, some useful information can be obtained from the large amount of data available from the main experiments conducted on the four plant species examined with the Idaho soil HAs. In particular: (a) sample HA4 promotes germination and early plant growth of western wheatgrass and basin wildrye at the highest concentrations and of bluebunch wheatgrass at the lowest concentrations, whereas it generally affects negatively germination and early growth of slickspot peppergrass; and (b) sample HA5 also promotes germination and early growth of western wheatgrass at the highest concentrations, promotes germination of bluebunch wheatgrass and basin at any concentration but has a concentration-differentiated effect on their early growth, and a contrasting concentration-effect on germination and a positive effect at the highest concentration on the early growth of slickspot peppergrass. The

different action of HA4 and HA5 on germination and early growth of the four plant species examined can be related only to their different C/N ratio and RFI.
Further, on slickpot peppergrass: (a) sample HA4-IIs at any concentration increases seedling primary root length and promotes early plant growth, but has a concentration-differentiated effect on germination % and seedling primary shoot length; (b) sample HA4-IIv at any concentration exerts a positive effect on germination % and root elongation but a depressing effect on shoot elongation; (c) sample HA4-IIc promotes germination % and root elongation at the highest concentrations with no or depressing effect at the lowest concentration, whereas the effcet on shoot elongation is concentration-differntiated. The different effects of these HAs on germination and early growth of slickspot peppergrass can be related to the differences in their C, H, N and O contents, C/N ratio, aliphatic, amide and carboxyl group composition, and RFI values.

LIST OF KEYWORDS

Slickspot peppergrass (*Lepidium papilliferum*), western wheatgrass (*Pseudoroegneria spicata*), bluebunch wheatgrass, basin wildrye (*Leymus cinereus*), seed germination, primary shoot, primary root, seedling early growth, shoots, roots, soils, Orthic Podzol, Elliot silt loam, Pahokee peat, leonardite, Idaho soils, silt in/out layer, vesicular in/out layer, clay in/out layer, humic acids, elemental composition, Fourier transform infrared (FT IR) spectroscopy, bi-dimensional emission-excitation (EEM) fluorescence spectroscopy, electron spin resonance (ESR) spectroscopy.

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STATEMENT OF THE PROBLEM AND BACKGROUND

Slickspot peppergrass (*Lepidium papilliferum*) is a reported “rare” ephemeral endemic plant found in the United States in Southwest Idaho’s high desert (Idaho Natural Heritage Program). *Lepidium* was designated in 1999 as a priority level "2" candidate for listing; a species with "high" threat magnitude, and "imminent" immediacy of threat, under the Endangered Species Act. (Federal Register 1999). Although the plant is reported as “rare” with “high" and "imminent" threat there is, little if any, scientific data to substantiate these claims. The United States (US) Air Force has an interest in *L. papilliferum* and effects on the Air Force mission in the event the plant becomes federally listed as threatened or endangered in the US.

L. papilliferum is known as “slickspot peppergrass” because it is associated primarily with slickspots and other wet areas. *L. papilliferum* coexists with slickspots that are scattered throughout the Southwest Idaho desert; thus, *L. papilliferum* has evolved in a heterogeneous fragmentized environment. The life cycle of *L. papilliferum* is uncertain. It appears that if there is enough moisture in the soil, most seeds sprout in March, April, and May. All the young plants first form a round "rosette" of bright green leaves. In late spring or early summer, some plants will bloom. Typically, these plants that bloom right away are annuals and have only one stem, and are very small, from 1 cm to 5 cm tall and from 1 cm to 2.5 cm in diameter, usually producing fewer than five flowers.

Nuttall first described what we now call *Lepidium papilliferum* as *Lepidium mountanum* (Torry and Gray 1838). Henderson (1900) further examined *L. mountanum* and classified it as *L. mountanum papilliferum.* Henderson reported “This plant I should call *L. papilliferum,* deeming it a good species, were it not that I find the pod quite variable in typical *L. mountanum,* the leaves running in some cases from pinnate to bi-pinnate, and very few papillae occasionally present on some parts of

the plant, even on Nuttall's type, with occasionally a very few *hairs* in place of papillae on the filaments but never papillae, as far as I have examined the materials in the Gray Herbarium. No. 4121". Nelson and Macbride (1914) made *L. mountanum papilliferum* a unique species, *L. papilliferum,* by concluding "it is distinguished at once from the species to which it has been referred by its distinctive habit of growth, its merely biennial duration and its unusual pubescence.

Today, however, *L. papilliferum* is reported to exist as a "biennial" or "annual" (Idaho Army National Guard 1998); although these terms may be inappropriate. Begon et al. (1990) call "annual" desert plants that do not always make seasonal appearances and have substantial seed banks that germinate after specific environmental events, "*semelparous ephemerals*". Based on *L. papilliferum* questionable classification history and lack of genetic information, the Air Force is interested in determining if *L. papilliferum* is a unique species or if it is a phenotypic morph of *L. mountanum* in an arid environment.

L. papilliferum establishes seed banks that retain viable dormant seeds for years. Meyer (1996) reported that after 4 years 60 to70% of original viable seeds were still viable and dormant. Pake and Venable (1996) examined plants in the Sonoran Desert and found many species maintain seed banks to buffer against unfavorable conditions. Data from Idaho Army National Guard Orchard Training Area's weather station, in association with a *L. papilliferum* population study, showed that spring precipitation had an important effect (Idaho Army National Guard 1998). In years with dry springs months, few *L. papilliferum* plants were present. In years with wet springs many plants were seen. Numbers of Orchard Training Areas *L. papilliferum* plants counted in an annual census varied from 7,000 in 1991 to 135,000 in 1995. Thus, the rarity of *L. papilliferum* appears to be more an expression of field sampling timing and local conditions in relation to spring moisture than actual scarcity.

Seed germination studies found that *L. papilliferum* seed stayed alive but dormant for several years, not germinating the first year even under many conditions of light and temperature. The seed can persist alive for at least four years in the soil. In one study, none of the *L. papilliferum* seed germinated the first winter, and only 10 to15% in each year thereafter. After four winters in the ground, 60 to 70% of the seeds were still alive and dormant. At this rate, there will still be living seeds in the ground for at least 7 to 11 years after they fall from the seed pods to the soil.

The objective of this research was thus to identifying and evaluating the effects of several naturally occurring humic acids (HAs) in Idaho soils on seed germination and seedling early-growth of slickspot peppergrass, and their possible relations to the compositional, structural and functional characteristics of HAs. A preliminary objective was to test three well characterized standard soil, peat and leonardite HAs and three additional plant species, western wheatgrass, bluebunch wheatgrass and basin wildrye.

METHODOLOGICAL APPROACH

MATERIALS

Plant Species

The plant seeds used were of four species, i.e., slickspot peppergrass (*Lepidium papilliferum*), western wheatgrass and bluebunch wheatgrass (*Pseudoroegneria spicata*) and basin wildrye (*Leymus cinereus*).

Humic Acids Used in the Preliminary Experiment

A preliminary germination and early plant growth experiment was performed in order to find the best experimental conditions for performing germination and early growth of the above elencated native american plant species object of this study, in the presence of various concentrations of four

humic acids (HAs) previously well characterized chemically and physico-chemically. One HA originated from an Orthic Podzol under a coniferous forest (OHA). The other three HAs were obtained from the Standard and Reference Collection of the International Humic Substances Society (IHSS), and originated from: (1) the mollic horizon of the Elliot silt loam soil sampled near Joliet, IL, USA, (SHA); (2) the Pahokee peat sampled at Ocachobee in the Everglades, FL, USA, (PHA); and (3) a leonardite sampled at the Gascoyne mine, Bowman County, ND, USA, (LHA).

Humic Acids Used in the Main Experiments

The main germination and early plant growth experiments were then performed using: (a) two HAs originated from two Idaho soils (sites # 4 and #5) (HA4 and HA5) object of this research (each HA sample was obtained by mixing three corresponding HA subsamples from three layers (0-1", 1-2", and 2-3") of the two Idaho soils); and (b) three HAs isolated from three mixed layers labeled "silt in/out", "vesicular in/out", and "clay in/out", of an Idaho soil (soil # 4, Series II) (HA4-IIs, HA4-IIv, and HA4-IIc) .

Humic Acid Selection for the Main Experiments

In order to select the HA samples appropriate for the main germination and early plant growth experiments, a total number of n. 61 HAs isolated from various layers of a number of Idaho soils were analysed for their chemical and spectroscopic properties (see below under Methods). These HAs were: (1) n. 7 HAs from three layers of Idaho sites # 4 and # 5, and one layer from site # 3; (2) n. 27 HAs from the layers labeled "silt-in", "vesicular-in", "clay-in", "silt-out", "vesicular-out", and "clay-out" of soils #1 to # 6 of Idaho soils Series I; and (3) n. 27 HAs from the layers labeled "silt-in", "vesicular-in", "clay-in", "silt-out", "vesicular-out", and "clay-out" of soils #1 to # 6 of Idaho soils Series II.

METHODS

Characterization of Humic Acids

The n. 61 HAs were chemically and physico-chemically characterized for their elemental (C, H, N, S, O) composition and structural and functional properties by using Fourier transform infrared (FT IR), bi-dimensional (emission-excitation matrix (EEM)) fluorescence and electron spin resonance (ESR) spectroscopies.

Preparation of Solutions of Humic Acids for the Experiments

The concentrations used for each HA in all germination and early growth experiments were: 10, 50, and 200 mg/L, with the exception of the preliminary experiment with OHA where concetrations used were: 10, 100, and 500 mg/L.
Each HA was first dissolved in few drops of KOH and then brought to the final volume with bidistilled water reaching a final pH ranging from 6.5 to 6.8.

Germination Experiments

In the **germination experiments**, ten (10) seeds of each species (western wheatgrass, bluebunch wheatgrass and basin wildrye) were placed in Petri dishes on filter paper, and added with solutions of each HA at each concentration (see above), or with bidistilled water (control). In the main experiments with HA4 and HA5, the Petri dishes were kept in the dark in a thermostated chamber

at the temperature of 24 °C for either 6 days (bluebunch wheatgrass), or 9 days (basin wildrye), or 10 days (western wheatgrass), so to obtain a complete germination. In the preliminary experiments with SHA, PHA and LHA, germination was performed as above but on a 10-day period for any plant seed. In the case of preliminary experiments with OHA, the seeds were kept in dark for 24 h at 5°C, then moved to the thermostated chamber where they were kept at 15°C for 48 h, and then at 26°C for either7 or 11 days, when the experiment ended. After these time periods, germinated seeds were removed and counted, and the lengths of the primary root and shoot were measured. All experiments were conducted in four replicates.

Tentative germination experiments made in the above-described conditions with **slickspot peppergrass** seeds were unsuccessful, thus a number of seed preliminary treatments and conditions were tested to improve germination. The treatments made in Petri dishes on n.30 seeds of each plant were: (a) soaking (SK) in distilled water for 24 h; (b) scraping (SC) with fine sand paper; (c) sterilizing (ST) with 0.2 % sodium hypochloride solution for 15 min; (d) piercing (PC) with steel needle; (e) shaking with 4 mL of suspended activated charcoal (AC) 10 mg/L for 1 h; (f) soaking in pure acetone (A) or 0.2 % (v/v) acetone/water for 15 min, then washing with distilled water; and (g) various combinations of the above. Only the treatment PC+SK was replicated 7 times for a total of n.210 seeds. The number of germinated seeds was counted after 7, 13, and 23 days.

Among the various tentative pretreatments described above to improve germination of slickspot peppergrass, the pretreatment selected and used here for the main germination experiment with **slickspot peppergrass** with HA4, HA5, HA4-IIs, HA4-IIv and HA4-IIc was the following:

The seeds were previously scraped dry with fine sand paper and then soaked in distilled water for 24 h before starting germination experiments. Thirthy (30) pretreated seeds of slickspot peppergrass were placed in Petri dishes on filter paper, and added with solutions of each HA at each concentration, or with bidistilled water (control). The Petri dishes containing the seeds were kept under diffuse light of tungsten-lamps in a thermostated chamber at a constant temperature of 25 °C for 5 days. No seed germinated in this period, thus all germinating seeds were pierced with steel needle and allowed to germinate for a longer time. After 6 days more, germinated seeds were removed and counted, and the lengths of the primary root and shoot were measured. All experiments were conducted in four replicates.

Early Growth Experiments

The **early-growth experiment** was started on germinated plant seeds (seedlings) immediately after the end of the germination experiment and after measuring the germination percentages and primary root and shoot lengths.

In the main experiments with Idaho soil HAs on western wheatgrass, bluebunch wheatgrass and basin wildrye the seedlings were inserted into holes of aluminum lids covering plastic pots (6 seedlings per pot) that were filled with the Nitch nutrient solution in the absence (control) or presence of each HA at three concentrations. The pH was adjusted with a solution of KOH to reach a value of 6.4. The pots were placed in a Fitotron growth chamber, and seedlings were allowed to grow for a period of 30 days in the following conditions: (a) photoperioed of 12 h; (b) temperature of 25°C; (c) humidity of 74%.

In the preliminary experiments with SHA, PHA and LHA, 4 seedlings per pot were used, the pH of the solutions was adjusted with NH_4OH to reach a vlue ranging from 5.9 to 6.5, and seedlings growth was conducted in the following conditions: (a) photoperiod of 12-h; (b) temperature of 23 °C and humidity of 70% during the illumination period; and (c) temperature of 17 °C and humidity of 65% during the dark period.

With OHA, the preliminary early-growth experiment was started after 19 days and was performed over 40 days, 5 seedlings per pot were used, and the growth conditions were: (a) photoperiod of 12 h; (b) temperature of 17 °C and humidity of 70% during the illumination period; and (c)

temperature of 23 °C and humidity of 65% during the dark period. At the end of the experiments, the pH of the solutions and length and fresh and dry weight of roots and shoots were measured. All experiments were conducted in four replicates.
In the experiment with **slickspot peppergrass**, germinated seedlings were transplanted into plastic pots containing cottonwool soaked with 8 mL of the Nitch nutrient solution in the absence (control) or presence of each HA at concentrations of 10, 50, and 200 mg/L. The pH of all solutions was previously adjusted at a value of 7.3 with a solution of KOH. The pots were covered with Parafilm and placed in a Fitotron growth chamber where seedlings were allowed to grow for a period of 24 days in the following conditions: (a) photoperiod of 12-h; (b) temperature of 25 °C during the illumination period and 20 °C during the dark period; and (b) constant humidity of 74%. At the end of the experiment, the length of roots and shoots were measured. All experiments were conducted in four replicates. It was not possible to measure the fresh and dry weights of shoots and roots because their amounts were very small.

Statistical Analysis of Experimental Data

All germination and early growth data were analysed statistically by one-way analysis of variance (ANOVA).

RESULTS AND DISCUSSION

HUMIC ACIDS

Elemental composition of n. 7 HA samples from Idaho soils of Sites # 4, # 5 and # 3
The elemental composition and atomic ratios are similar for the six HAs from sites # 4 and # 5, and fall within the range of values commonly reported for soil HAs. However, the C and H contents of the HAs slightly decrease with soil depth for both sample series #4 and #5, whereas the opposite occurs for O content. The C/N atomic ratios of HAs from soils #4 are slightly smaller than those of HAs from soils #5, and do not vary with soil depth. The N and S contents and the C/H and O/C ratios do not show any trend.
The FT IR spectra are also similar for the six HAs from sites # 4 and # 5, and similar to those commonly obtained for soil HAs. However, a slight decrease of intensity of aliphatic bands and amide II band may be observed with increasing depth for both HA series.
The EEM fluorescence spectra and maximum excitation/emission wavelength pairs (EEWPs) are also similar for the six HAs studied. The relative fluorescence intensity (RFI) is different for the HAs of the two series, and in both cases it increases with soil depth.
The ESR spectra of the six HAs studied are similar in showing a typical signal ascribed to organic free radical (OFR) species indigenous in HAs and typical resonance patterns of HA-complexed Cu (II) ions. The concentration of OFR in HAs from soils #4 decreases with depth, whereas that of HAs from soils #5 do not not show any trend.
The elemental composition and atomic ratios of HA sample from site #3, depth 0-1 inches, the FT IR spectrum, emission/excitation matrix fluorescence spectrum, the ESR spectrum, the maximum excitation/emission wavelength pair (EEWP), the relative fluorescence intensity (RFI), and the organic free radical concentrations (OFR), are similar to those of the six HAs from sites #4 and #5, and fall within the range of values commonly reported for soil HAs.

Elemental composition of n. 27 HA samples from Idaho soils Series I
The elemental composition (C, H, N, S, O) and calculated atomic ratios on a moisture- and ash-free basis of the n. 27 HAs from Series I soils indicate that the content of each element measured and atomic ratios are within values typical of soil HAs. Although no trend is apparent as a function of soil type, the soil layer appears to exert some influence on the elemental composition of HAs. In

particular, in any soil type, C, H, and N contents appear to generally decrease, and O content and C/N ratio increase, when passing from the silt to the vesicular to the clay layer for both "in" and "out" HAs.
The FT IR spectra of the n.27 HAs investigated are generally similar one to another, and to those commonly obtained for soil HAs. However, the relative intensity of aliphatic group absorption (at about 2930 and 2855 cm^{-1}) and amide C=O absorption (amide I, 1541 cm^{-1}) decrease and that of carboxyl group absorptions (about 1716 and about 1230 cm^{-1}) increase, in the order, for HAs isolated from: silt-in layer, vesicular-in layer, clay-in layer. Almost no difference is apparent between corresponding "in" and "out" layers. Further, most samples show a broad and intense band at about 1100 cm^{-1} which can be ascribed to silicate impurities in HAs.
The EEM fluorescence spectra and EEWPs of the n.27 HAs investigated indicate that the EEWP are quite similar for all the HAs investigated, although some slight differences are observable but with no specific trend. The relative fluorescence intensity (RFI) is different for the HAs investigated and generally tends to increase in the order: silt, vesicular, clay, either "in" and "out" layers.

Elemental composition and spectroscopic characterization of n. 27 HA samples from Idaho soils Series II
The elemental composition (C, H, N, S, O) and calculated atomic ratios on a moisture- and ash-free basis of the n. 27 HAs from Series II soils also indicate that the element contents and atomic ratios are within the range typical of soil HAs. Although no trend is apparent as a function of soil type, the soil layer appears to exert some influence on the elemental composition of HAs. In particular, in any soil type, C, H, and N contents appear to generally decrease, and O content and C/N ratio increase, when passing from the silt to the vesicular to the clay layer for both "in" and "out" HAs.
The FT IR spectra of the n.27 HAs investigated are generally similar one to another, to the corresponding ones of Series I HAs, and to those commonly obtained for soil HAs. However, the relative intensity of aliphatic group absorption (at about 2930 and 2855 cm^{-1}) and amide C=O absorption (amide I, 1541 cm^{-1}) decrease and that of carboxyl group absorptions (about 1716 and about 1230 cm^{-1}) increase, in the order, for HAs isolated from: silt-in layer, vesicular-in layer, clay-in layer. Almost no difference is apparent between corresponding "in" and "out" layers. Further, most samples show a broad and intense band at about 1100 cm^{-1} which can be ascribed to silicate impurities, possibly to the large ash content of several HAs.
The EEM fluorescence spectra and EEWPs of the n.27 HAs investigated indicate that the EEWPs are quite similar for all the HAs investigated and similar to EEWPs of the corresponding HAs of Series I, although some slight differences are observable but with no specific trend. The relative fluorescence intensity (RFI) is different for the HAs of Series II investigated here, and differs from RFI value of the corresponding HAs of Series I. The RFI generally tends to increase in the order: silt, vesicular, clay, either "in" and "out" layers.

GERMINATION AND EARLY PLANT GROWTH

General Comments on Germination and Early Plant Growth Results

Germination and early-growth results obtained on: (a) seeds of western wheatgrass and bluebunch wheatgrass (*Pseudoroegneria spicata*) and basin wildrye (*Leymus cinereus*) both in the preliminary experiments with OHA, SHA, PHA and LHA and in the main experiments with HA4 and HA5 (any HA used at three concentrations); and (b) seeds of slickspot peppergrass (*Lepidium papilliferum*) in the main experiments with HA4 and HA5, HA4-IIs, HA4-IIv, HA4-IIc at three concentrations, are very variable and often contrasting as a function of both the seed species and the HA type and

concentration. These results will be summarized and discussed in the following text using the plant species as the main variable.

Statistical analysis by one-way variance of all experimental dataobtained for each parameter measured distinctly show, with respect to the corresponding controls, and as a function of either the type or the concentration of HAs used, the existence of: (a) no significant difference in germination percentages (%), length of primary shoot and root growth of germinated seeds and length and fresh and dry weights of shoots and roots of western wheatgrass, bluebunch wheatgrass and basin wildrye grown for 30 days, in the case of samples OHA, SHA, PHA and LHA; (b) generally significant (0.05 P) or highly significant (0.01 P) differences for all measured parameters in the case of HA4 and HA5 in experiments with western wheatgrass, bluebunch wheatgrass and basin wildrye; (c) no significant difference in germination percentages, primary shoot length and shoots length after 24-days of growth and a highly significant difference (0.01 P) for the length of primary root of germinated seeds and roots after 24-days of growth, in the case of HA4 and HA5 in experiments with slickspot peppergrass; (d) highly significant (0.01 P) or significant (0.05) differences for all measured parameters, in the case of HA4-IIs, HA4-IIv and HA4-IIc in experiments with slickspot peppergrass.

Western Wheatgrass

Effect of HA type and concentration on germination

Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) OHA produces a decrease of germination % at concentrations of 10 and 100 mg/L and an increase at 500 mg/L, whereas primary shoot and root length increase at any concentration; (b) SHA at any concentration used has no effect on germination % and primary shoot length, whereas primary root length is not affected at a concentration of 10 mg/L, is increased at 50 mg/L, and is decreased at 200 mg/L; (c) PHA and LHA at any concentration used have a slight promoting effect on germination % and a depressing effect on primary shoot length, whereas LHA at any concentration depresses primary root length and PHA does not affect primary root length at a concentration of 200 mg/L, affects it positively at 50 mg/L and negatively at 10 mg/L; (d) HA4 at concentrations of 50 and 200 mg/L exerts a slightly positive effect and at 10 mg/L a marked negative effect on germination %, whereas it appears to have no effect on primary shoot and root length; (e) HA5 affects positively the germination % at a concentration of 200 mg/L but negatively at 10 and 50 mg/L, promotes primary shoot length at concentrations of 50 and 200 mg/L and primary root length at 200 mg/L, and has no effect on primary shoot length at 10 mg/L and primary root length at 10 and 50 mg/L.

Effect of HA type and concentration on early growth

Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) SHA exerts a promoting effect on the length and fresh and dry weights of shoots at 200 mg/L and of roots at 50 and 200 mg/L, and no effect or a slight depressing effect at the other concentrations; (b) PHA promotes roots length at concentrations of 50 and 200 mg/L and shoots length at 10 and 50 mg/L but has no effect at the other concentrations, increases markedly the fresh and dry weights of roots at any concentration, whereas the shoots fresh weight increases only at 10 mg/L but decreases at 200 mg/L, and the shoots dry weight decreases at any concentration; (c) LHA at any concentration has no effect on shoots length and a promoting effect on roots length at 50 and 200 mg/L, decreases fresh shoots length and increases fresh roots length at any concentration, and increases dry shoots length at 50 and 200 mg/L and dry roots length at 50 mg/L showing no or deceasing effects at the other concentrations; (d) HA4 has no effect on shoots length but a promoting effect on roots length at 50 and 200 mg/L and a depressing effect on both at 10 mg/L, slightly increases fresh shoots weight at 50 mg/L and fresh roots weight at 200 mg/L but decreases both at 10 mg/L and has no effect at the other concentrations, and has no effect on shoots dry weight at 50 and 200 mg/L and on roots dry weight at 200 mg/L but increases roots dry weight at 50 mg/L and decreases both at 10

mg/L; (e) HA5 at any concentration has no effect on shoots length and a promoting effect on roots length, no effect on fresh weights and positive effect on dry weights of shoots and roots at at 10 and 200 mg/L and a decreasing effect on both at 50 mg/L.

Bluebunch Wheatgrass

Effect of HA type and concentration on germination
Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) OHA produces an increase of germination % at a concentration of 10 mg/L and a decrease at 100 and 500 mg/L, whereas primary shoot and root length increase slightly at any concentration; (b) SHA at any concentration used has a slightly positive effect on germination %, almost no effect on primary shoot length, whereas primary root length is depressed at concentrations of 10 and 50 mg/L, and not affected at 200 mg/L; (c) PHA at any concentration used has a slight promoting effect on germination % and a promoting effect on primary shoot and root length at a concentration of 50 mg/L but no effect at 10 and 200 mg/L; (d) LHA at any concentration used has a slight promoting effect on germination %, no effect on primary shoot length and depresses primary root length; (d) HA4 at concentrations of 10 and 50 mg/L has no effect and at 200 mg/L a marked negative effect on germination %, whereas primary shoot and especially root lengths increase at 10 and 50 mg/L, and are not affected at 200 mg/L; (e) HA5 at any concentration used affects positively the germination % and, especially, primary shoot and root lengths.

Effect of HA type and concentration on early growth
Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) SHA at any concentration used exerts no effect on shoots length and roots dry weight, and a promoting effect on roots length and, especially, roots fresh weigth, whereas shoots fresh and dry weigths increase at concentrations of 10 and 50 mg/L but decrease at 200 mg/L; (b) PHA at any concentration promotes roots length and has no effect on shoots length, increases markedly the fresh and dry weights of roots at 50 and 200 mg/L but decreases or has no effect at 10 mg/L, whereas the fresh and dry weights of shoots increase at 200 mg/L but decrease at 10 and 50 mg/L; (c) LHA at any concentration has almost no effect on shoots and roots lengths, increases fresh and dry roots weight at any concentration and fresh and dry shoots length at 200 mg/L but has almost no effect at 10 and 50 mg/L; (d) HA4 has no or depressing effect on shoots and roots lengths at concentrations of 50 and 200 mg/L but a promoting effect, especially on roots length, at 10 mg/L, slightly increases fresh shoots weight at 10 mg/L and fresh roots weight at 50 mg/L but decreases both at 200 mg/L and has no effect at the other concentrations, and increases shoots dry weight at 10 and 50 mg/L and roots dry weight at 10 and 200 mg/L but decrease shoots dry weight at 200 mg/L and has no effect on root dry weight at 50 mg/L; (e) HA5 at any concentration has a differentiated promoting effect on shoots and roots lengths, decreases or has no effect on the shoots fresh and dry weights at 10 and 200 mg/L but increases both at 50 mg/L, decreases roots fresh weight at any concentration, and increases roots dry weights at 10 and 50 mg/L but decreases it at 200 mg/L.

Basin Wildrye

Effect of HA type and concentration on germination
Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) OHA produces no effect on germination % at concentrations of 10 and 100 mg/L and a decrease at 500 mg/L, whereas primary shoot and root length increase slightly at 500 mg/L but decrease at 10 and 100 mg/L; (b) SHA at any concentration used has a slightly negative or no effect on germination % and a positive effect on primary root length, whereas primary shoot length is increased at concentrations of 50 and 200 mg/L but depressed at 10 mg/L; (c) PHA at any concentration used

has a slight negative or no effect on germination %, and a promoting effect on primary shoot and root lengths at a concentration of 10 mg/L but a depressing or no effect at 50 and 200 mg/L; (d) LHA at any concentration used has a slightly negative or no effect on germination % and a slightly positive effect or no effect on primary root length, whereas primary shoot length is positively affected at a concentration of 200 mg/L but not affected at 10 and 50 mg/L; (d) HA4 at concentrations of 10 and 50 mg/L has no effect and at 200 mg/L a marked positive effect on germination %, whereas primary shoot and root lengths increase at any concentration; (e) HA5 at any concentration used affects positively the germination % and primary shoot and root lengths.

Effect of HA type and concentration on early growth
Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) SHA at any concentration used exerts no effect on shoots length and fresh and dry weights and a slightly depressing effect on roots lengths, whereas roots fresh and dry weights increase at a concentration of 10 mg/L but decrease at 50 and 200 mg/L; (b) PHA at any concentration has no effect on roots length but promotes shoots fresh and dry weights, has no effect on shoots length at concentrations of 10 and 50 mg/L but a promoting effect at 200 mg/L, increases roots fresh weight at 50 and 200 mg/L but has no effect at 10 mg/L, and increases roots dry weight at 10 and 200 mg/L but depresses it at 50 mg/L; (c) LHA at any concentration has almost no effect on shoots length but depresses roots length and has a positive effect on shoots and roots dry weights, increases shoots fresh weight at a concentration of 200 mg/L but has no effect at 10 and 50 mg/L, and has a slightly positive or no effect on roots fresh weight; (d) HA4 at any concentration exerts a promoting effect on roots length and no effect on roots dry weight, at 50 and 200 mg/L has no effect on shoots length and fresh weight and a promoting effect on shoots dry weight but a general depressing effect at 10 mg/L, and has no effect on roots fresh weight at 50 mg/L but a depressing effect at 10 and 200 mg/L; (e) HA5 at any concentration has a differentiated promoting effect on the length and, especially, fresh and dry weights of shoots and length and dry weight of roots, whereas almost no effect is exterted on roots fresh weight.

Slickspot peppergrass

Preliminary germination experiments

In general, a small number of slickspot peppergrass seeds germinated after 7, 13, and 23 days in the various experimental conditions preliminary tested to improve germination, and described above. The higher number of germinated seeds were obtained after 13 days in the following conditions: (a) PC wet + SK (11 seeds germinated); (b) SC dry + SK (10 seeds germinated); and (c) SC dry (9 seeds germinated). The other treatments appear to induce limited germination in the first 7 days, which then stops. Only the SK experiment produced a late (23 days), very small (2 seeds) germination. Thus, the seeds in the main experiment were finally germinated in the conditions also described above whose results are described in the following text.

Effect of HA type and concentration on germination
Statistical analysis of numerical data obtained suggest that, with respect to the control: (a) HA4 slightly increases germination % at concentrations of 10 and 50 mg/L but decrease it at 200 mg/L, and affects negatively the primary shoot and root lengths at any concentration; (b) HA5 slightly increases germination % at a concentration of 50 mg/L but does not affect it at 10 mg/L and decreases it at 200 mg/L, and has almost no effect on primary shoot length at any concentration and on primary root length at 10 and 50 mg/L but increases markedly the latter at 200 mg/L; (c) HA4-IIs promotes germination % at 10 and 200 mg/L but has no effect at 50 mg/L, increases primary shoot length at 50 mg/L but has no effect at 10 and 200 mg/L, and increases primary root length at any concentration; (d) HA4-IIv at any concentration promotes germination %, slightly

increases primary root length, and decreases primary shoot length; (e) HA4-IIc promotes germination % at 50 and 200 mg/L but has no effect at 10 mg/L, has no effect on primary shoot and root lengths at 200 mg/L but decreases both at 10 and 50 mg/L.

Effect of HA type and concentration on early growth

However, statistical analysis of numerical data obtained for shoots and roots lengths after 24-days of growth suggest that, with respect to the control: (a) HA4 at any concentration exerts a negative effect on shoots and roots lengths; (b) HA5 at any concentration has no effect on shoots length, and a marked positive effect on roots length at a concentration of 200 mg/L but no effect at 10 and 50 mg/L; (c) HA4-IIs at any concentration increases shoots and roots length; (d) HA4-IIv has no effect on shoots length at 10 and 50 mg/L but decrease it at 200 mg/L, and increases roots length at any concentration; (e) HA4-IIc has no effect on shoots length at 10 and 200 mg/L but decreases it at 50 mg/L, and increase roots length at 200 and 50 mg/L but has no effect at 10 mg/L.

CONCLUSIONS AND RECOMMENDATIONS

In general, germination and early growth results obtained both in the preliminary and main experiments are quite variable, and sometime contrasting, on dependence of the kind of seed and seedling and origin and concentration of HA used. Although no definite trend can be observed for the germination and early growth parameters measured, some useful information that can be obtained from the large amount of data available from the main experiments conducted on the four plant species examined with the Idaho soil HAs will be illustrated in the following text.

In particular: (a) sample HA4 promotes germination and early plant growth of western wheatgrass and basin wildrye at the highest concentrations and of bluebunch wheatgrass at the lowest concentrations, whereas it generally affects negatively germination and early growth of slickspot peppergrass; and (b) sample HA5 also promotes germination and early growth of western wheatgrass at the highest concentrations, promotes germination of bluebunch wheatgrass and basin at any concentration but has a concentration-differentiated effect on their early growth, and a contrasting concentration-effect on germination and a positive effect at the highest concentration on the early growth of slickspot peppergrass. The different action of HA4 and HA5 on germination and early growth of the four plant species examined can be related only to their different C/N ratio and RFI.

Further, on slickpot peppergrass: (a) sample HA4-IIs at any concentration increases seedling primary root length and promotes early plant growth, but has a concentration-differentiated effect on germination % and seedling primary shoot length; (b) sample HA4-IIv at any concentration exerts a positive effect on germination % and root elongation but a depressing effect on shoot elongation; (c) sample HA4-IIc promotes germination % and root elongation at the highest concentrations with no or depressing effect at the lowest concentration, whereas the effcet on shoot elongation is concentration-differntiated. The different effects of these HAs on germination and early growth of slickspot peppergrass can be related to the differences in their C, H, N and O contents, C/N ratio, aliphatic, amide and carboxyl group composition, and RFI values.

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APPENDIXES

APPENDIX 1

LIST OF PUBLICATIONS RELATED TO THIS RESEARCH

T.J. Cary, A.J. Palazzo, T.L. Bashore, N. Senesi, G. Brunetti, C.E. Clapp, J.J. Clapp, M.H.B. Hayes, K. Schmidt-Rohr, J.D. Mao. Isolation and characterization of humic acids in soil collected at juniper butte range, Idaho. Proc. 20th Ann. Conf. IHSS (IHSS 12), July 2004, São Pedro, 175-177 (2004).

E. Loffredo, A.J. Palazzo, N. Senesi, and C.E. Clapp. 2004. Seed germination and seedling early growth of three native american species as affected by soil, peat and leonardite humic acids under controlled conditions in climatic chamber. ASA-CSSA-SSSA Annual Meeting Abstracts, Paper n. 3491.

R.S. Vial, C.E. Clapp, J.J. Clapp, T.J. Cary, A.J. Palazzo, N. Senesi, G. Brunetti, M.H.B. Hayes, K. Schmitt-Rohr, and J.D. Mao. 2004. Characterization of humic acids from Idaho "slickspot" soils. ASA-CSSA-SSSA Annual Meeting Abstracts, Paper n. 5712.

APPENDIX 2

LIST OF ALL PARTICIPATING SCIENTIFIC PERSONNEL SHOWING ANY ADVANCED DEGREES EARNED BY THEM WHILE EMPLOYED ON THE PROJECT

Dr. Elisabetta Loffredo, earned the degree of Associate Professor from Assistant Professor.
Dr. Giuseppe Ferrara, earned the degree of Assistant Professor.

APPENDIX 3

Only data obtained in the last three month period of this research , and not presented in the previous five interim Reports, are included in Appendix 3.

Table 1. Ash content, elemental composition and atomic ratios of humic acids (HAs)

Sample	Ash	C	H	N	S	O	C/N	C/H	O/C
	($g\ kg^{-1}$) [a]	($g\ kg^{-1}$) [b]					(Atomic ratios)		
6-25-I	178	555	60	66	5	314	9.8	0.8	0.4
6-26-I	368	553	45	54	4	344	12.0	1.0	0.5
6-27-I	137	548	46	47	4	355	13.7	1.0	0.5
1-1-II	596	561	66	55	3	315	12.0	0.7	0.4
1-2-II	283	557	53	51	4	335	12.8	0.9	0.5
1-3-II	94	551	51	48	5	346	13.4	0.9	0.5
2-7-II	279	547	50	45	3	354	14.2	0.9	0.5
3-12-II	34	557	50	46	3	344	14.1	0.9	0.5
6-25-II	274	553	65	62	4	316	10.3	0.7	0.4
6-26-II	82	553	51	56	5	335	11.5	0.9	0.5
6-27-II	90	534	47	45	3	371	13.9	1.0	0.5

[a] On moisture-free basis.
[b] On moisture- and ash-free basis.

Table 2. Significance level (F value) resulting from one-way Analysis of Variance (ANOVA) of all data obtained for each parameter measured for slickspot peppergrass.

Parameter	
Germination	2.48 *
Primary shoot length	4.30 **
Primary root length	2.23 *
Shoots length	3.73 **
Roots length	3.26 **

** 0.01 P; * 0.05 P.

Table 3. Effect of HAs at different concentrations on seed germination (percentage of germinated seeds ± standard error for four replicates) of slickspot peppergrass measured immediately before transplanting.

Treatment	
Control (H_2O)	8.3 ± 1.9
HA4-IIs 10 mg/L	11.7 ± 1.9
50 mg/L	8.3 ± 1.9
200 mg/L	10.8 ± 0.7
HA4-IIv 10 mg/L	13.3 ± 2.0
50 mg/L	13.3 ± 2.0
200 mg/L	10.8 ± 1.4
HA4-IIc 10 mg/L	8.3 ± 1.9
50 mg/L	16.7 ± 1.7
200 mg/L	18.3 ± 3.0

Table 4. Effect of HAs at different concentrations on the length (cm ± standard error for four replicates) of primary shoot and primary root of germinated seeds of slickspot peppergrass.

Treatment	Shoot	Root
Control (H_2O)	0.63 ± 0.07	0.96 ± 0.05
HA4-IIs 10 mg/L	0.65 ± 0.06	1.29 ± 0.22
50 mg/L	1.01 ± 0.02	1.44 ± 0.03
200 mg/L	0.60 ± 0.08	1.70 ± 0.50
HA4-IIv 10 mg/L	0.48 ± 0.07	1.06 ± 0.26
50 mg/L	0.47 ± 0.11	1.06 ± 0.05
200 mg/L	0.48 ± 0.10	1.03 ± 0.20
HA4-IIc 10 mg/L	0.40 ± 0.09	0.80 ± 0.02
50 mg/L	0.40 ± 0.05	0.87 ± 0.15
200 mg/L	0.59 ± 0.05	0.98 ± 0.05

Table 5. Effect of HAs at different concentrations on the length (cm ± standard error for four replicates) of shoots and roots of slickspot peppergrass measured after 24-day growth.

Treatment	Shoot	Root
Control (H_2O)	0.81 ± 0.10	1.27 ± 0.08
HA4-IIs 10 mg/L	0.92 ± 0.11	1.60 ± 0.20
50 mg/L	1.42 ± 0.29	1.75 ± 0.19
200 mg/L	0.89 ± 0.14	2.11 ± 0.41
HA4-IIv 10 mg/L	0.84 ± 0.09	1.74 ± 0.32
50 mg/L	0.80 ± 0.11	1.65 ± 0.17
200 mg/L	0.64 ± 0.17	1.56 ± 0.13
HA4-IIc 10 mg/L	0.76 ± 0.03	1.21 ± 0.32
50 mg/L	0.67 ± 0.05	1.42 ± 0.13
200 mg/L	0.82 ± 0.07	1.70 ± 0.13

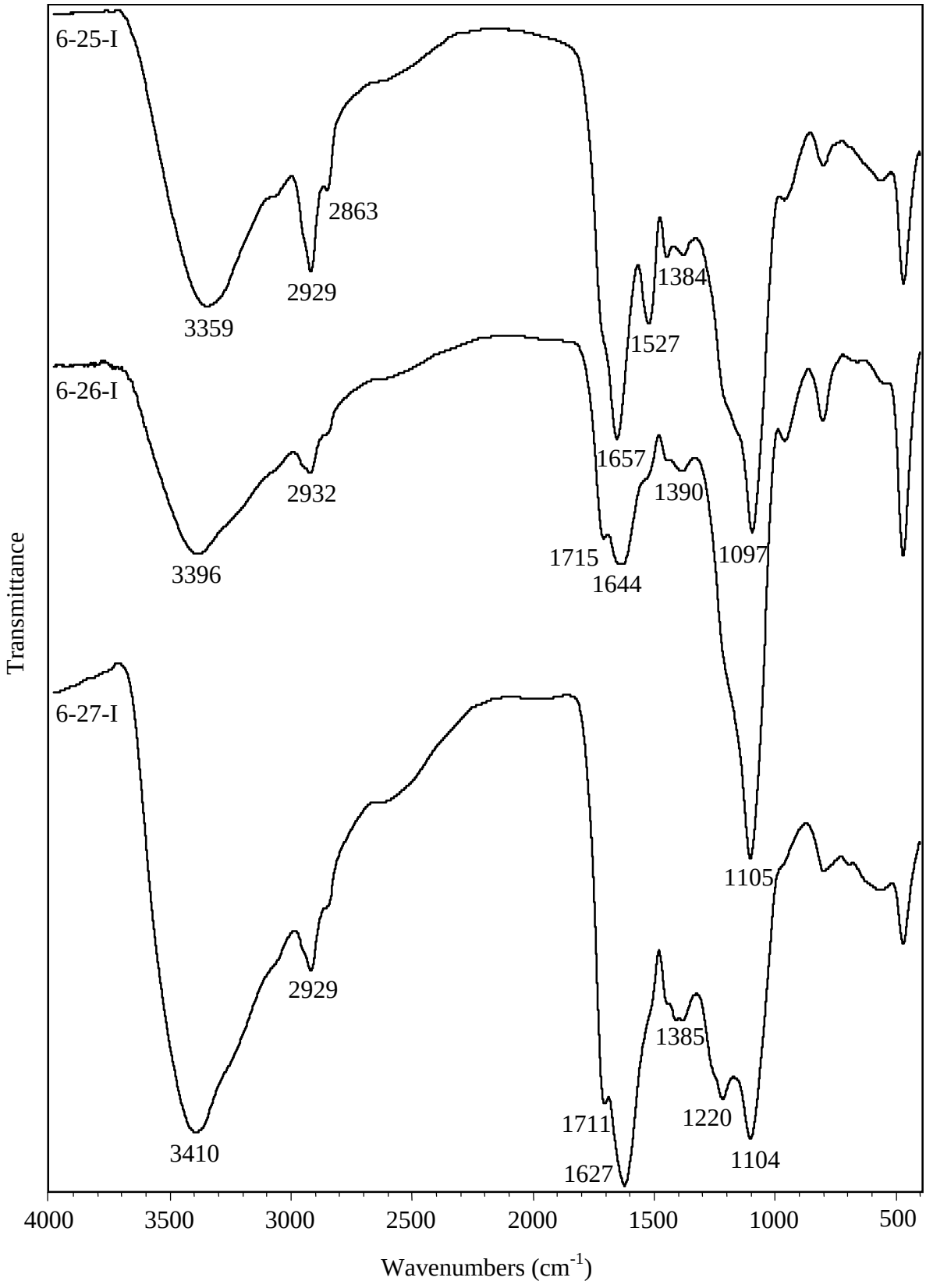

Figure 1. Fourier transform infrared (FT IR) spectra of humic acids.

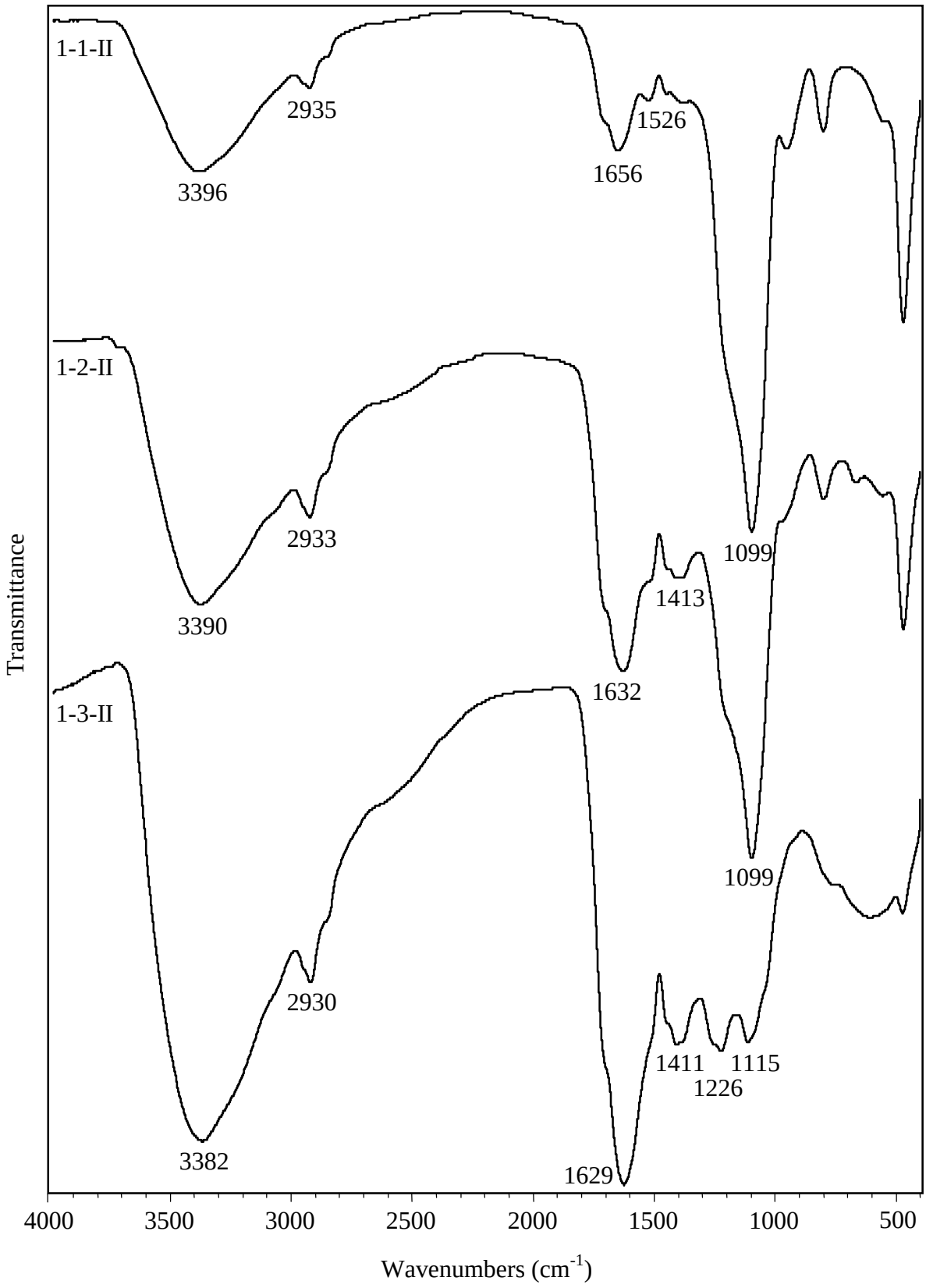

Figure 2. Fourier transform infrared (FT IR) spectra of humic acids.

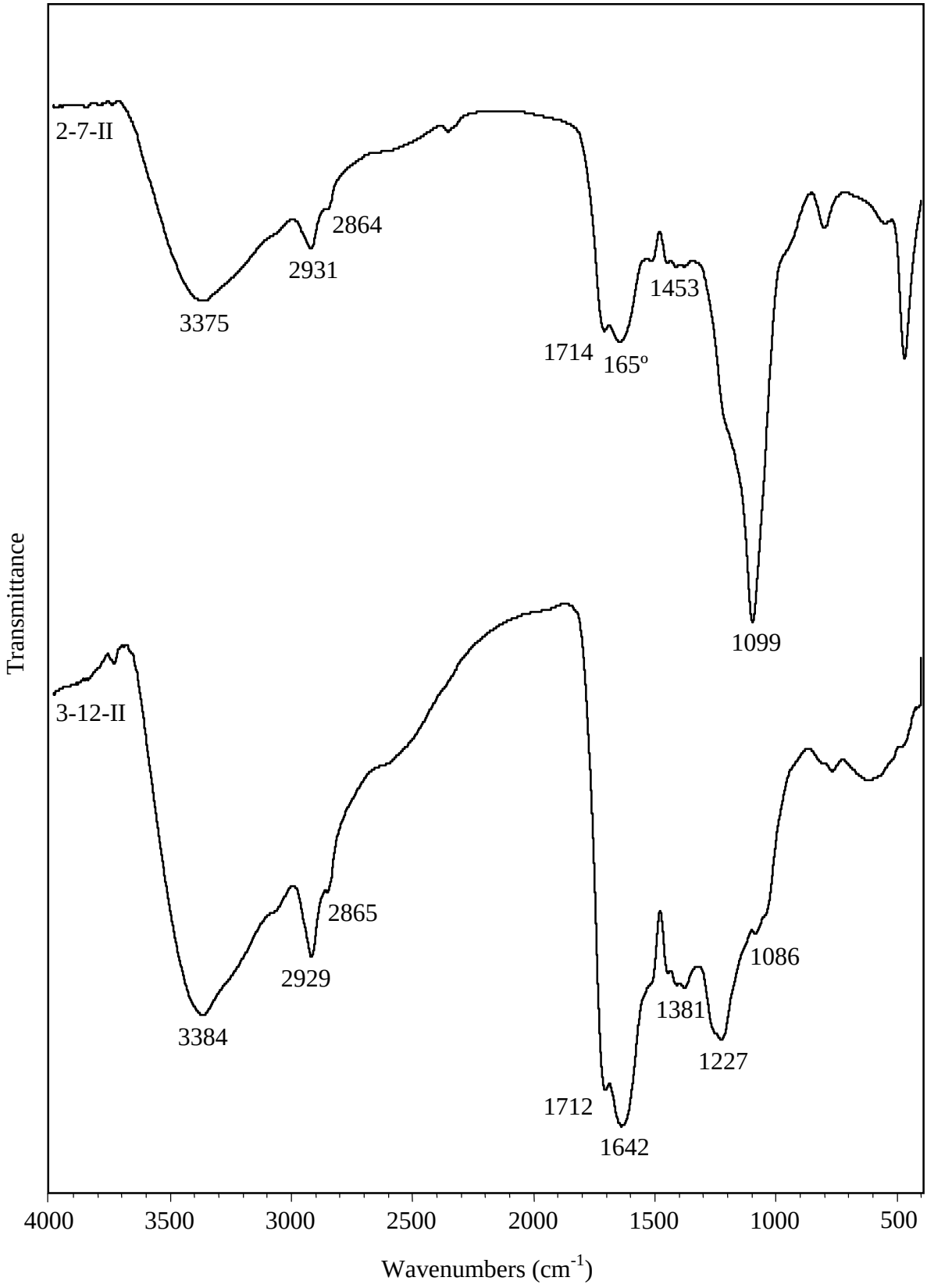

Figure 3. Fourier transform infrared (FT IR) spectra of humic acids.

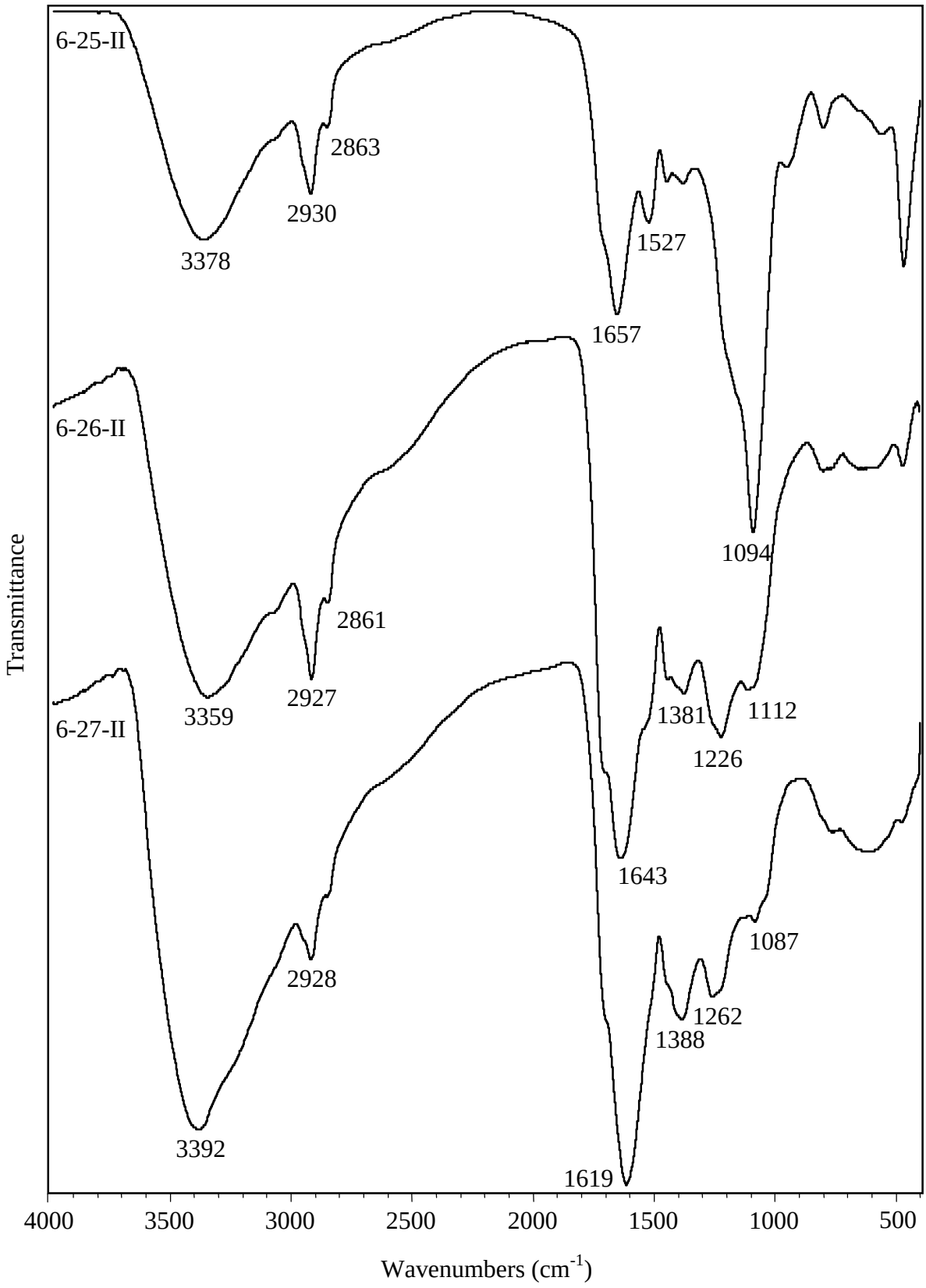

Figure 4. Fourier transform infrared (FT IR) spectra of humic acids.

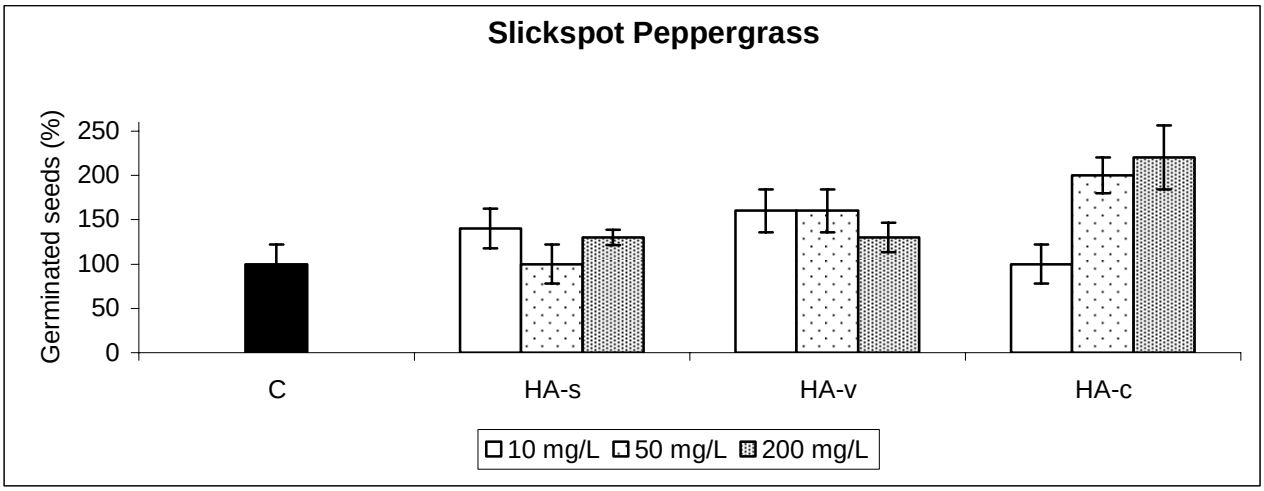

Figure 5. Effect of HAs at different concentrations on the number of germinated seeds expressed as percentages of control treatment (100 %). The vertical line on each bar indicates the standard error for 4 replicates.

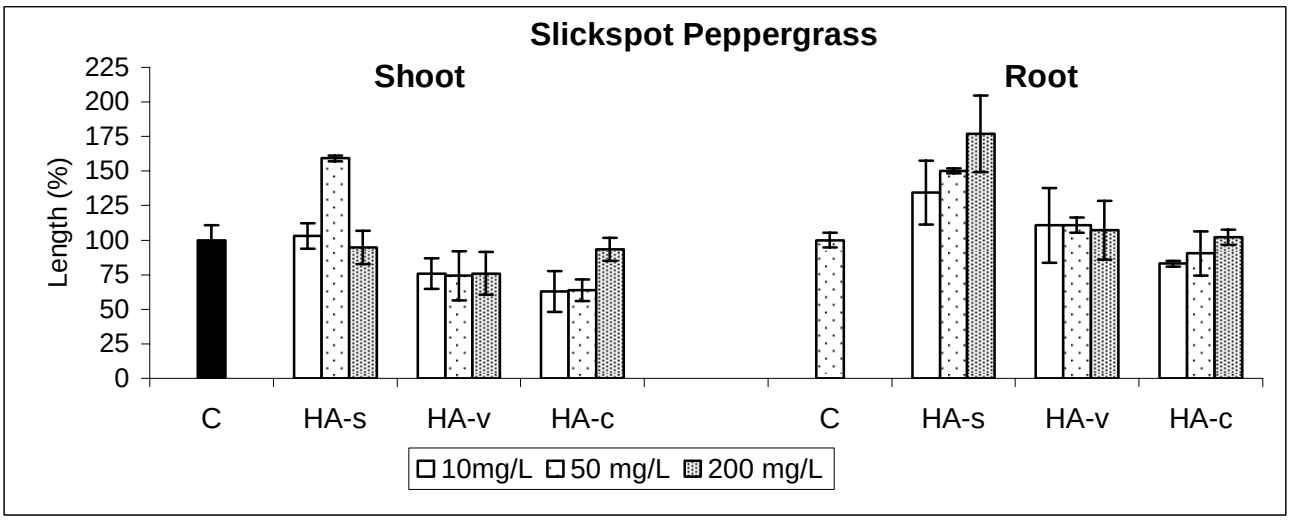

Figure 6. Effect of HAs at different concentrations on primary shoot and root length of germinated seeds, expressed as percentages of control treatment (100 %). The vertical line on each bar indicates the standard error for 4 replicates.

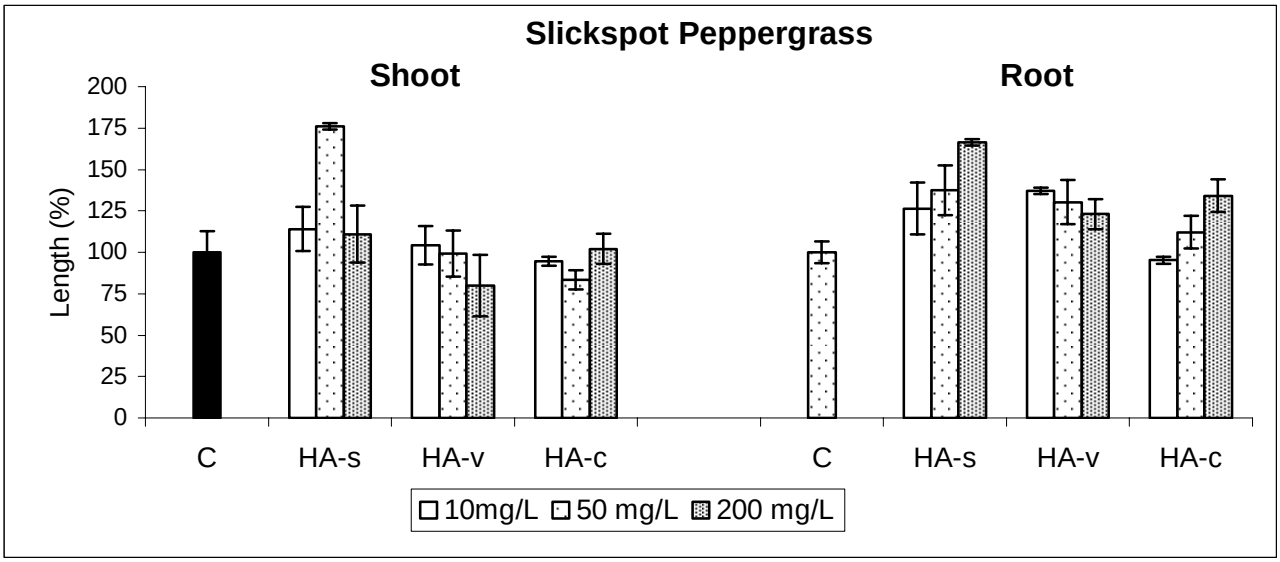

Figure 7. Effect of HAs at different concentrations on the length of shoots and roots expressed as percentages of control treatment (100 %) measured after 21-day growth. The vertical line on each bar indicates the standard error for 4 replicates.

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